Infrared Spectroscopic Characterization of the Structural Changes Connected with the $E_1 \rightarrow E_2$ Transition in the $\text{Ca}^{2+}$-ATPase of Sarcoplasmic Reticulum*

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The $\text{Ca}^{2+}$-transporting ATPase (EC 3.6.1.38) of sarcoplasmic reticulum alternates between several conformational states during ATP-dependent $\text{Ca}^{2+}$ transport. The $E_1$ conformation is stabilized by 0.1 mM $\text{Ca}^{2+}$ and the $E_2$ conformation by vanadate in a $\text{Ca}^{2+}$-free medium. Fourier transform infrared spectroscopy reveals significant differences between the two states that indicate differences in the protein secondary structure. The two states and the corresponding spectra can be interconverted reversibly by changing the $\text{Ca}^{2+}$ concentration of the medium. The infrared spectral changes indicate the appearance of a new $\alpha$-helical substructure connected with the $E_1 \rightarrow E_2$ conversion accompanied by small changes in $\beta$-turns, while the $\beta$-sheet content remains essentially unchanged. There are also differences between the $E_1$ and $E_2$ states in the C=O stretching vibrations of the ester carbonyl groups of phospholipids in intact sarcoplasmic reticulum that are not observed under identical conditions in isolated sarcoplasmic reticulum lipid dispersions. These observations imply an effect of proteins on the structure of the interfacial regions of the phospholipids that is dependent on the conformational state of the $\text{Ca}^{2+}$-ATPase. The CH$_2$ and CH$_3$-stretching frequencies of the membrane lipids are not affected significantly by the $E_1 \rightarrow E_2$ transition. The Fourier transform spectra of sarcoplasmic reticulum vesicles in the presence of 20 mM $\text{Ca}^{2+}$ suggest the stabilization of a protein conformation similar to the $E_2$ state except for differences in the behavior of COO$^-$ and phospholipid ester C=O groups that may reflect charge effects of the bound $\text{Ca}^{2+}$.

The $\text{Ca}^{2+}$-transporting ATPase (EC 3.6.1.38) is an intrinsic membrane protein that constitutes about 80% of the protein content of isolated sarcoplasmic reticulum vesicles (Martonosi and Beeler, 1983). The predicted structure of Ca$^{2+}$

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1 The abbreviations used are: FT-IR, Fourier transform infrared; EGTA, [ethylenebis(oxyethyl enenitrilo)tetraacetic acid.

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sarcoplasmic reticulum and in reconstituted Ca\textsuperscript{2+}-ATPase vesicles (Mendelsohn et al., 1984; Jaworsky and Mendelsohn, 1985; Anderle and Mendelsohn, 1986) reveal interesting selectivity in the interaction of Ca\textsuperscript{2+}-ATPase with certain phospholipid classes.

The focus of this report is the effect of Ca\textsuperscript{2+}, EGTA, and vanadate on the Amide I and II vibrations arising from protein peptide bonds and on the C=O stretching vibrations of the lipid ester carbonyl group in rabbit sarcoplasmic reticulum vesicles and in isolated sarcoplasmic reticulum lipids. The data indicate distinct differences in protein secondary structures between the E\textsubscript{1} and E\textsubscript{2} conformations of Ca\textsuperscript{2+}-ATPase and a clear relationship between the structure of Ca\textsuperscript{2+}-ATPase and the conformation of the lipid ester carbonyl bond.

**EXPERIMENTAL PROCEDURES**

**Materials**

Deuterium oxide was obtained from MSD isotopes, Merck Chemical Division, St. Louis, MO 63116. EGTA, phosphonolipoytrypate, \(\beta\)-nicotinamide adenine dinucleotide, lactic dehydrogenase (rabbit muscle), pyruvate kinase (rabbit muscle), and adenosine 5'-triphosphate were supplied by Sigma. A23187 was purchased from Behring Diagnostics, and sodium vanadate was obtained from Fisher. All other reagents were of analytical grade.

**Methods**

**Preparation of Sarcoplasmic Reticulum**—The sarcoplasmic reticulum vesicles were isolated from predominantly white skeletal muscles of rabbits as described earlier (Jona and Martonosi, 1986), and the preparations were stored before use frozen at -70°C in 0.5 M sucrose, 10 mM Tris-maleate, pH 7.0, at a protein concentration of 25–38 mg/ml.

About 16–18 h before measurements, 1- to 2 ml aliquots of the microsomes were thawed, centrifuged, and the pellet resuspended in 5 ml of standard medium containing 0.1 M KCl, 10 mM imidazole, pH 7.4, and 1 mM MgCl\textsubscript{2} in either H\textsubscript{2}O or D\textsubscript{2}O. The centrifugation and resuspension steps were repeated twice more. The final sediments were taken up in standard H\textsubscript{2}O or D\textsubscript{2}O medium to a final protein concentration of 24–33.6 mg/ml.

FT-IR measurements were carried out: (a) without further addition, (b) after the addition of CaCl\textsubscript{2} to a final concentration of 0.1 mM, (c) after addition of CaCl\textsubscript{2} to a final concentration of 20 mM, and (d) after addition of 1 mM EGTA and 5 mM Na\textsubscript{2}VO\textsubscript{4}.

Concentrated stock solutions of CaCl\textsubscript{2}, EGTA, and vanadate were prepared in H\textsubscript{2}O or D\textsubscript{2}O containing standard medium.

**Protein** was determined according to Lowry et al. (1951).

**Extraction of Phospholipids from Sarcoplasmic Reticulum**—The preparation of sarcoplasmic reticulum phospholipids was carried out essentially as described by Folch et al. (1957). The extracted lipids were dried under nitrogen and suspended in standard H\textsubscript{2}O or D\textsubscript{2}O medium. After ultrasonic dispersion the liposomes were stored at -70°C in D\textsubscript{2}O medium. A tungsten-copper thermocouple was taped directly onto the window and the cell placed into a thermostated cell mount.

**RESULTS**

**Infrared Spectroscopy of Sarcoplasmic Reticulum Vesicles in H\textsubscript{2}O and in D\textsubscript{2}O**—The spectra of sarcoplasmic reticulum membranes were taken up in standard H\textsubscript{2}O or D\textsubscript{2}O medium to a final protein concentration of 0.1 M KCl, 0.02 M Tris-HCl, pH 7.5, 0.9 mM CaCl\textsubscript{2}, 5 mM MgCl\textsubscript{2}, 0.42 mM phosphonoacrylate, 0.2 mM NADH, 0.45 IU of pyruvate kinase, and 18 IU of lactate dehydrogenase at 3°C for 2 h. A23187; the micromolar protein concentration was 1 mg/ml. After preincubation 1 ml of medium II was added to start the reaction. Medium II contained 0.1 M KCl, 0.02 M Tris-HCl, pH 7.5, 1 mM EGTA, 5 mM MgCl\textsubscript{2}, 10 mM Ca\textsuperscript{2+}, and phosphonoacrylate, NADH, pyruvate kinase, and lactate dehydrogenase at the same concentration as in medium I. The preincubination in medium I was required to displace vanadate from the binding site of the Ca\textsuperscript{2+}-ATPase. The absorbance was measured at 340 nm at 25°C using a Perkin-Elmer Lambda 3-B dual wavelength spectrophotometer. The recorded absorbance was linear up to at least 10–15 min of reaction time. For measurement of the Ca\textsuperscript{2+}-insensitive (basal) ATP hydrolysis, Ca\textsuperscript{2+} was omitted from the medium. The Ca\textsuperscript{2+}-insensitive ATP hydrolysis rate was usually less than 5% of the rate of ATP hydrolysis in Ca\textsuperscript{2+}-containing medium. Data on total ATP hydrolysis rates measured in Ca\textsuperscript{2+}-containing media were corrected for the Ca\textsuperscript{2+}-insensitive ATPase activity.

**Fourier Transform Infrared Spectroscopy**

Infrared spectra of aqueous vesicle suspensions were recorded on a Digilab FTS-15 FT-IR spectrometer using a demountable cell (Harrick Scientific, Ossining, NY) with calcium fluoride windows and 6-μm spacers for samples in H\textsubscript{2}O medium or 50-μm spacers for samples in D\textsubscript{2}O medium. A tungsten-copper thermocouple was taped directly onto the window and the cell placed into a thermostated cell mount. For each spectrum 512 interferograms were averaged at an optical retardation of 0.5 cm, triaxially apodized, and Fourier transformed to yield FT-IR spectra with a resolution of 2 cm\textsuperscript{-1}. The H\textsubscript{2}O or D\textsubscript{2}O spectra were digitally subtracted using a spectrum of the medium as a reference. In order to separate instrumentally unresolvable infrared bands, Fourier second-derivative and Fourier derivation techniques (Noffat et al., 1986) were applied. Band narrowing by Fourier deconvolution was performed by using Lorentzian bandwidths of 15 cm\textsuperscript{-1} and a resolution enhancement factor of 2.2. Fourier derivation was performed by using a power of 3 and a breakpoint of 0.5. A fourth derivative corresponds to a smoothed fourth derivative. The ATPase activity of sarcoplasmic reticulum samples was measured before and after recording the FT-IR spectra, and the activities did not change significantly.

**REFERENCES**

The unique advantage of infrared spectrometry is that it allows the simultaneous study of the structure of lipids and proteins of intact biological membranes under near physiological conditions without the introduction of reporter groups. While the strong water absorption in the region 1700–1600 cm\textsuperscript{-1}, where the structurally most significant protein bands are located, presents difficulties in the early applications of infrared spectrometry to biological systems, modern FT-IR spectrometers generate infrared spectra with a signal-to-noise ratio high enough to subtract the water component from the protein spectrum. The problem caused by the H\textsubscript{2}O band may also be prevented by the use of D\textsubscript{2}O instead of H\textsubscript{2}O as solvent; however, under these conditions the replacement of exchangeable hydrogens by deuterium leads to changes in the spectrum that must be taken into account in the analysis and interpretation of the data.

**Infrared Spectra of Sarcoplasmic Reticulum Vesicles in \textit{H}_2\textit{O} and in \textit{D}_2\textit{O**—The spectra of sarcoplasmic reticulum mem}
branes isolated from rabbit muscle are shown in Fig. 1 after solvent subtraction in H$_2$O (solid line) and in D$_2$O solutions (dashed line). Several differences can be seen. The maximum of the Amide I band which is at 1652 cm$^{-1}$ in H$_2$O buffer has shifted to 1646 cm$^{-1}$ in D$_2$O. This is due to H-to-D exchange of the amide N-H groups in um (or less) ordered structures that changes the overall band shape. The Amide II band shifts from 1550 cm$^{-1}$ in H$_2$O to 1460 cm$^{-1}$ after deuteration; a residual Amide II band that is retained in D$_2$O medium at 1550 cm$^{-1}$ is due to the slow exchange of amide hydrogens in ordered structures and in domains that are sheltered from solvent exchange. Furthermore, the decrease in the intensity of the Amide II band in a D$_2$O medium allows one to see other vibrations that are usually hidden by the strong Amide I band in H$_2$O; among these are the side chain vibrations of amino acids, such as carboxylate and carbimino groups and the C-C stretching vibrations of the phenyl group of tyrosine (Chirgadze et al., 1975).

The Amide I and II modes generate unique band contours in the region of 1800-1500 cm$^{-1}$. Generally, both the Amide I and II band contours consist of several component bands which overlap each other. Because of the intrinsic widths of these component bands (their half-bandwidth, typically 20-25 cm$^{-1}$, is larger than the separation between individual bands), they cannot be resolved by increased instrumental resolution. In the past, this fact had severely limited the application of infrared spectroscopy to protein conformational analysis. More recently mathematical methods have been developed that allow the computational narrowing of infrared bands that comprise a complex band contour. While this operation is often referred to as resolution enhancement, it does not increase the instrumental resolution but increases the degree to which individual component bands can be visualized. Currently, two such computational procedures are used for band narrowing, Fourier deconvolution and derivation. Fourier deconvolution is an iterative procedure controlled by two adjustable parameters, i.e. the bandwidth and a resolution enhancement factor. The Fourier derivation is an alternative and mathematically different method of spectral resolution enhancement that is based on the generation of "non-integer derivative" band profiles; the degree of resolution enhancement is under the control of a single "break-point" parameter which can be adjusted. The validity of these procedures was tested on synthetic curves (Kauppinen et al., 1981), and both techniques are now being used extensively to resolve overlapping infrared bands in complex systems, such as lipids (Casal and Mantsch, 1984; Mendelsohn and Mantsch, 1986) and proteins (Lee et al., 1985; Renugopalakrishnan et al., 1985; Byler and Susi, 1986; Olinger et al., 1986; Jaworsky et al., 1986; Haris et al., 1986).

Changes in the Infrared Spectra of Sarcoplasmic Reticulum Connected with the Transition between the E$_1$ and E$_2$ Conformations of the Ca$^{2+}$-ATPase—The Ca$^{2+}$-ATPase of sarcoplasmic reticulum is stabilized in the E$_1$ conformation by 0.1 mM CaCl$_2$ and in the E$_2$ conformation by 1.0 mM EGTA + 5 mM vanadate. Fig. 24 shows the spectra of sarcoplasmic reticulum dispersed in D$_2$O medium in the two conformational states. The naked eye is unable to detect significant differences between the two spectral curves. However, subtle differences became apparent after suitable data processing, as evident from Fig. 2, B and C, which show the same spectra after band narrowing, respectively, by Fourier deconvolution or Fourier derivation. In each case the solid line corresponds to sarcoplasmic reticulum in the presence of 0.1 mM Ca$^{2+}$ (E$_1$)

![Infrared spectra of sarcoplasmic reticulum in H$_2$O and D$_2$O.](image1.png)

**Fig. 1.** Infrared spectra of sarcoplasmic reticulum in H$_2$O and D$_2$O. Solid line, spectrum of sarcoplasmic reticulum vesicles (24 mg of protein/ml) in H$_2$O medium containing 0.1 M KCl, 10 mM imidazole, pH 7.4, 1 mM MgCl$_2$, and 0.1 mM CaCl$_2$, after subtraction of the spectra of the H$_2$O medium without sarcoplasmic reticulum. The spectra were recorded at 10°C in a CaF$_2$ cell of 6 μm thickness and corrected for water vapor. Broken line, spectra of sarcoplasmic reticulum vesicles (24 mg of protein/ml) in a D$_2$O medium of otherwise identical composition, measured at 10°C in a BaF$_2$ cell of 50 μm thickness, after subtraction of the spectrum of D$_2$O medium without sarcoplasmic reticulum and correction for water vapor. In both cases the recording started 1 h after addition of Ca$^{2+}$ to the sarcoplasmic reticulum vesicles dispersed in the appropriate standard medium.

![Original, deconvolved, and derivative spectra of sarcoplasmic reticulum in D$_2$O medium in the E$_1$ and E$_2$ states.](image2.png)

**Fig. 2.** Original, deconvolved, and derivative spectra of sarcoplasmic reticulum in D$_2$O medium in the E$_1$ and in the E$_2$ states. A, to sarcoplasmic reticulum vesicles (24 mg of protein/ml) suspended in a standard medium of 0.1 M KCl, 10 mM imidazole, pH 7.4, and 1 mM MgCl$_2$, either 0.1 mM CaCl$_2$ (solid line) or 1 mM EGTA and 5 mM monovanadate (broken line) were added to stabilize the E$_1$ and E$_2$ conformations, respectively. The spectra were taken at 10°C about 1 h after the additions in a 50-μm BaF$_2$ cell. From both spectra the spectrum of D$_2$O medium and the contribution of water vapors were subtracted. B, same spectra as in A after resolution enhancement by Fourier deconvolution, using a band narrowing factor of 2.2. C, same spectra as in A after band narrowing by Fourier derivation using a power of 3 and a breakpoint of 0.4.
state), and the dashed line in the presence of 1 mM EGTA + 5 mM vanadate (E2 state). Our practice in assessing the validity of resolution-enhanced band profiles is to generate them independently by two different procedures and compare the results. Only peaks that show up in both procedures are considered. Thus, from a comparison of the resolution-enhanced spectra we are confident that all spectral features observed as individual bands in Fig. 2B or as shoulder bands in Fig. 2C are real and represent specific conformational structures. As can be seen from Fig. 2B, the degree of band narrowing is less pronounced upon deconvolution; however, the intensities of the component bands (integrated band areas) are preserved. Upon derivation (Fig. 2C) sharper peaks are obtained, but the true line shape is lost, and the peak intensity becomes dependent on the width of the original bands. For example, the peak at 1516 cm⁻¹ has the greatest intensity in the derivative spectra because it is narrower than the other bands, although it is only a small peak in the original spectrum (see Fig. 1). The advantage of derivation over deconvolution is that it allows a more precise determination of the position of component bands that appear only as shoulder bands in the deconvolved spectra.

The region 1800–1500 cm⁻¹ contains bands coming from the peptide backbone of the protein, from the amino acid side chains, and from the lipid moieties of sarcoplasmic reticulum. The following regions have been chosen to analyze the conformational differences in sarcoplasmic reticulum between the E1 and E2 states: (a) 1700–1620 cm⁻¹, which corresponds to the Amide I band, that is most sensitive to conformational changes, (b) 1655–1535 cm⁻¹, which contains the Amide II band, (c) 1620–1650 cm⁻¹, with bands related to amino acid side chain vibrations, and (d) 1760–1700 cm⁻¹ that gives information about the phospholipid headgroup.

The Amide I Region (1700–1620 cm⁻¹)—Fig. 3 shows the resolution enhanced infrared spectra of sarcoplasmic reticulum in D₂O in the presence of 0.1 mM calcium that stabilizes the E1 state (solid line), 1 mM EGTA + 5 mM vanadate that stabilizes the E2 state (broken line), or 20 mM calcium without definite conformational assignment (dotted line). The Amide I bands observed in the 1700–1620 cm⁻¹ region of the infrared spectra of Ca²⁺-ATPase in the E1 and E2 conformations are given in Table I, along with their assignment to specific secondary substructures. Whereas the spectra obtained in the presence of 5 mM vanadate or 20 mM calcium are very similar, there are differences between these and the spectrum obtained in the presence of 0.1 mM calcium. The most important of these differences is the presence of a peak at 1650 cm⁻¹ in the E2 state stabilized by vanadate, that is absent in the E1 state, stabilized by 0.1 mM CaCl₂. Surprisingly, the 1650 cm⁻¹ peak reappears when the [Ca²⁺] is raised to 20 mM. The binding of Ca²⁺ to the low affinity Ca²⁺-binding sites of the Ca²⁺-ATPase apparently stabilizes a conformation that is similar to the E2 state induced by vanadate.

The peak at 1650 cm⁻¹, specific of the E2 state, occurs in the Amide I region characteristic for α-helices and probably represents a new α-helical structure that may arise from the rearrangement of other structures during the E₁ → E₂ transition. The appearance of the new 1650 cm⁻¹ band in the E2 state may occur at the expense of the 1657 cm⁻¹ or the 1643 cm⁻¹ bands.

In the spectra of both the E1 and E2 conformations, the band at 1657 cm⁻¹ can be assigned to α-helices, and the bands at 1630 and 1682 cm⁻¹ to antiparallel β-sheet structures (Susi et al., 1967; Cortijo et al., 1982; Mendelsohn et al., 1984; Arrondo et al., 1985; Lee et al., 1985; Jaworsky et al., 1986). The infrared bands at 1668 and 1692 cm⁻¹ are attributable to turns (Krimm and Bandekar, 1980). There is one additional band in the spectrum of the E1 state of Ca²⁺-ATPase at 1677 cm⁻¹ which is absent in the spectrum of the E2 state and which most likely also represents turns. Alternatively, the peak at 1677 cm⁻¹ may correspond to the high frequency component of a β-sheet structure (Susi et al., 1967); this is, however, less likely since there is no doubling of the corresponding low frequency component at 1630 cm⁻¹ that is much stronger in intensity. In the spectra obtained in the presence of 20 mM calcium or 5 mM vanadate, the two peaks at 1683 and 1677 cm⁻¹ have apparently merged into one composite peak due to a change in the position or a decrease in the intensity of one of the bands. It is more likely that the band representing turns is affected. The band at 1643 cm⁻¹ is assigned to unordered structures. In H₂O medium (not shown), the band assigned to unordered structures overlaps with the α-helix band, giving rise to a composite band at 154.5 cm⁻¹.

The Amide II Region (1565–1535 cm⁻¹)—The Amide II and Amide I bands represent different vibrational modes; whereas the Amide I mode is a C=O stretching vibration, the Amide II mode is mainly a N–H bending vibration (Susi, 1969, 1972; Mendelsohn and Mantsch, 1986). Despite some attempts to

| Amide I bands observed in the 1700–1620 cm⁻¹ region of the infrared spectra of Ca²⁺-ATPase in the E₁ and E₂ conformations and their assignment |
|---|---|
| E₁ state | E₂ state |
| 1630 (β) | 1630 (β) |
| 1643 (R) | 1643 (R) |
| 1650 (α) | 1650 (α) |
| 1657 (α) | 1657 (α) |
| 1668 (T) | 1668 (T) |
| 1677 (T/β) | 1677 (T/β) |
| 1683 (β) | 1682 (β) |
| 1692 (T) | 1692 (T) |

Infrared Spectroscopy of Sarcoplasmic Reticulum
correlate the Amide II band with the same structural patterns as established for the Amide I band, no conclusive experimental results have been obtained. The position of the Amide II band is sensitive to deuteration, shifting from around 1550 cm\(^{-1}\) to the so-called Amide II' frequency at 1450 cm\(^{-1}\). The Amide II' band overlaps with the H-O-D bending vibration, so it has no value in the analysis of conformational changes. Fig. 1 shows clearly the decrease in Amide II intensity upon deuteration (H \(\rightarrow\) D exchange of the N-H groups). However, the remainder of the Amide II band at 1550 cm\(^{-1}\) is a clue to the accessibility of solvent to the peptide backbone. Hydrophobic environments or tightly ordered structures (\(\alpha\)-helix or \(\beta\)-sheet) prevent the amide H from being exchanged. In Fig. 4 the residual Amide II bands of the two conformational states (\(E_{1}\) and \(E_{2}\)) of sarcoplasmic reticulum are compared. It is evident that in D\(_{2}\)O medium only the peak at 1550 cm\(^{-1}\) is left in the \(E_{1}\) state, whereas there is a second Amide II peak at 1537 cm\(^{-1}\) in the \(E_{2}\) state. This suggests that at least some of the groups which were accessible to H \(\rightarrow\) D exchange in the \(E_{2}\) conformation are protected in the \(E_{1}\) state. The vanadate-induced changes characteristic of the \(E_{2}\) state are reversed on addition of 0.1 mM CaCl\(_{2}\) that restores the spectral characteristics of the \(E_{1}\) state.

Amino Acid Side Chain Vibrations (1630–1560 cm\(^{-1}\)) — In the region 1580–1500 cm\(^{-1}\), in addition to protein bands attributed to the peptide backbone, some bands due to side chain vibrations are also seen (Chirgadze et al., 1975). Little is known about these vibrations and their conformational sensitivity. The 1516 cm\(^{-1}\) band due to the C-C stretching of the phenolic tyrosine ring (Fig. 2, B and C) is apparently not affected by protein conformational changes. It is interesting to note that in the region 1620–1650 cm\(^{-1}\), where the side chain vibrations of charged amino acids occur, the spectrum of the 0.1 mM calcium sample is closer to that of the 20 mM calcium sample than to that of the sample in 5 mM vanadate. It is plausible to assume that the charged residues (i.e. the -COO\(^{-}\) groups) are more sensitive to the type of cation bound than to changes in protein conformation. The bands due to sarcoplasmic reticulum protein side chain vibrations in H\(_{2}\)O and in D\(_{2}\)O media are similar. Some bands around 1700 cm\(^{-1}\) may correspond to the nonionized forms of the amino acids.

Phospholipid Headgroup Vibrations (1760–1700 cm\(^{-1}\)) — The region 1760–1700 cm\(^{-1}\) contains vibrations from the ester carbonyl groups of the membrane phospholipids. In model systems two bands can be distinguished in this region that correspond to the sn-1 and sn-2 C=O groups (Bush et al., 1980; Levin et al., 1982). In natural membranes the interpretation may be less straightforward, since several phospholipid classes with different acyl chains are normally present and even some amino acid side chain vibrations can be encountered in this region. Fig. 5A shows the deconvolved spectra of sarcoplasmic reticulum in the presence of 0.1 mM calcium (solid line), 20 mM calcium (dotted line), and 5 mM vanadate. In all three cases there are two peaks at 1741 and 1731 cm\(^{-1}\) that can be assigned to the sn-1- and sn-2-ester carbonyls, respectively. The ratio between the 1741 and 1731 peaks is greater than 1 in the presence of 0.1 mM calcium (\(E_{1}\)) and less than 1 in the presence of vanadate (\(E_{2}\)). There are two additional peaks at 1750 cm\(^{-1}\) and 1717 cm\(^{-1}\) in the 5 mM vanadate and in the 0.1 mM calcium samples that are not seen in the 20 mM calcium spectrum.

In order to establish whether the differences observed in Fig. 5A are due to the protein or to a direct effect of calcium on the phospholipids, the phospholipids were extracted from sarcoplasmic reticulum and their spectra compared in the presence of 0.1 mM calcium, 5 mM vanadate, or 20 mM calcium (Fig. 5B). The 1741 cm\(^{-1}\) peak is observed more or less in the same position; however, in the 20 mM Ca\(^{2+}\) spectrum (and to a lesser extent in the 0.1 mM Ca\(^{2+}\) spectrum) the 1731 cm\(^{-1}\) peak has split in two. The bands at 1750 cm\(^{-1}\) and at 1717 cm\(^{-1}\) which are clearly seen in the spectra of sarcoplasmic reticulum in the presence of 0.1 mM CaCl\(_{2}\) or 5 mM vanadate (Fig. 5A) become weak shoulders in the corresponding lipid extracts (Fig. 5B).

From these data it is clear that there is an influence of the protein on the ester carbonyl groups of phospholipids, since the lipids exhibit different infrared spectra in the presence or in the absence of protein. It is not possible to attribute this to a direct effect of Ca\(^{2+}\) on phospholipids, since no large differences are seen under identical conditions in the extracted lipid. The splitting found in the lipid extract at high calcium concentration is not observed when there is protein

![Fig. 5. Infrared spectra of sarcoplasmic reticulum (A) and of the extracted sarcoplasmic reticulum lipids (B) in H\(_{2}\)O medium under various experimental conditions after Fourier deconvolution (C=O stretching modes). A, sarcoplasmic reticulum vesicles (24 mg of protein/ml) were suspended in standard H\(_{2}\)O medium and supplemented with 0.1 mM CaCl\(_{2}\) (solid line), 1 mM EGTA, and 5 mM sodium vanadate (broken line) or 20 mM CaCl\(_{2}\) (dotted line). Spectra were taken at 8.8–10 °C and the spectrum of H\(_{2}\)O medium subtracted. B, sarcoplasmic reticulum lipid extracts were extracted as described under "Experimental Procedures" and suspended in standard H\(_{2}\)O medium to a final concentration of 53 mg/ml. Additions were made and spectra recorded as described under "Experimental Procedures."](https://example.com/fig5.png)
in the system. Although some buffering of calcium by proteins may occur, the differences observed between the E₁ and E₂ states in sarcoplasmic reticulum in the vibrations assigned to the ester carbonyl bonds most probably reflect the influence of protein on phospholipids, and this influence is different when the Ca²⁺-ATPase is in the E₁ as compared with the E₂ state. The precise nature of the conformation-dependent effect of Ca²⁺-ATPase on the ester carbonyl vibration of phospholipids is not known, but it is likely to involve the stalk region (Brandl et al., 1986) of the Ca²⁺-ATPase that is close to the water-lipid interface.

The PO₂-stretching vibrations are not changed, which is consistent with the proposition that the differences in the carbonyl ester stretching vibrations are not due to a direct effect of the cations on the phospholipids, but reflect an influence of the protein.

The infrared spectra of delipidated sarcoplasmic reticulum (not shown) indicate gross structural changes (decrease of α-helix content and increase of β-sheet and unordered structures) connected with the removal of phospholipids. Based on the intensity of Amide II band the H → D exchange is more pronounced in native than in delipidated sarcoplasmic reticulum.

**The Acyl Chains of Phospholipids—**Another spectral region of interest is the 3000-2800 cm⁻¹ range where the bands due to the methyl- and methylene-stretching vibrations are seen; these are related to the conformational arrangement of the acyl chains (Casal and Mantsch, 1984). In native sarcoplasmic reticulum membranes at 8-10°C, the acyl chains are in the liquid crystalline state, which is thought to be necessary to sustain the enzymatic activity of the Ca²⁺-ATPase. There is no difference in the CH₂- or CH₃-stretching band frequencies between the E₁ or E₂ states.

**Temperature-dependent Changes in the Structure of Sarcoplasmic Reticulum—**Proteins undergo a temperature-induced denaturation that involves a major rearrangement of the tertiary structure. A nearly complete H → D exchange is observed in D₂O medium upon denaturation (Englander and Kallenbach, 1984). This is also evident from the loss of the Amide II band at 1550 cm⁻¹ upon thermal denaturation (Fig. 6A). Furthermore, new peaks appear in the Amide I region of the infrared spectra that indicate a loosening of structures. After thermal denaturation of the sarcoplasmic reticulum in D₂O, the new peaks at 1619 and 1686 cm⁻¹ dominate the Amide I band, and there is a decrease in the intensity at 1658 and 1630 cm⁻¹. The broad peak centered at 1648 cm⁻¹ probably represents primarily unordered structures together with some remnants of the secondary structures that were present in the native protein before thermal denaturation.

Fig. 6B compares the spectra of sarcoplasmic reticulum in D₂O in the presence of either 0.1 mM calcium or 1 mM EGTA + 5 mM vanadate after thermal denaturation. In the Amide I region, the two spectra are similar with peaks located at 1688, 1648, and 1619 cm⁻¹; the Amide II is completely exchanged in both cases. In the 1700-1700 region, two peaks are visible in the presence of 0.1 mM calcium, while the 5 mM vanadate sample has only one broad band. The midpoint of denaturation temperature is 42°C in the E₁ and 50°C in the E₂ state. Therefore, 1 mM EGTA and 5 mM vanadate slightly stabilize the Ca²⁺-ATPase against thermal denaturation. Under similar conditions vanadate also increased the stability of Ca²⁺-ATPase against inactivation by 1-2 kbar pressure (Varga et al., 1986) suggesting a more compact structure in the E₁ than in the E₂ state. The similar profiles of thermally denatured sarcoplasmic reticulum in the presence of 0.1 mM calcium or 1 mM EGTA + 5 mM vanadate show that the two different conformational states (E₁ and E₂) can only exist when the native tertiary structure is present.

**DISCUSSION**

The FT-IR spectra of sarcoplasmic reticulum stabilized by 0.1 mM CaCl₂ in the E₁ conformation and by vanadate in the E₂ conformation show significant differences. These differences indicate changes in the secondary structure of the protein and in the conformation of the phospholipid ester carbonyl group related to the transition between the E₁ and E₂ states. The CH₂ and CH₃ stretching vibrations of the acyl chains of phospholipids are not affected by the transition, and there are no clearly identified changes in vibrations related to amino acid side chains in the sarcoplasmic reticulum proteins. Since the Ca²⁺-ATPase constitutes at least 80% of the protein content of sarcoplasmic reticulum, the Ca²⁺ and vanadate induced changes in protein secondary structure are likely to occur in the Ca²⁺-ATPase rather than in the accessory proteins of sarcoplasmic reticulum. Nevertheless, conclusive evidence on this point is not available and may be very difficult to obtain. Reconstituted vesicle preparations containing purified Ca²⁺-ATPase have been widely used, but recent x-ray and neutron diffraction data indicate that the disposition of Ca²⁺-ATPase in reconstituted vesicles is very different from that in the native sarcoplasmic reticulum (Herbette et al., 1983); therefore, it is difficult to relate with confidence structural information obtained on reconstituted Ca²⁺-ATPase vesicles to the native membranes. Ca²⁺-induced changes in the secondary structure of calsequestrin have been reported (for review see MacLennan et al., 1983); however, a recent Raman spectroscopic study (Williams and Beeler, 1986) casts
some doubt on these observations. In any case, calsequestrin is a minor component representing less than 5% of the protein content of sarcoplasmic reticulum used in these studies; therefore, it is unlikely to affect significantly the FT-IR spectrum. Based on these considerations, it seems justified to assume that the observed changes in protein secondary structure involve the Ca\textsuperscript{2+}-ATPase.

The E\textsubscript{1} conformation stabilized by 0.1 mM Ca\textsuperscript{2+} contains less α-helix than the E\textsubscript{2} conformation stabilized by EGTA + vanadate. This is in essential agreement with earlier observations of Arrondo et al. (1985) that Ca\textsuperscript{2+} decreases the helix content of sarcoplasmic reticulum, compared with an EGTA-containing, i.e. Ca\textsuperscript{2+}-free, system. There is no detectable difference in β-sheet and a slight difference in β-turn content between the E\textsubscript{1} and E\textsubscript{2} conformations. These data represent the first clear indication that changes in the secondary structure of Ca\textsuperscript{2+}-ATPase accompany the previously observed rearrangement of structural domains within the ATPase molecule connected with the E\textsubscript{1} → E\textsubscript{2} transition (Dux et al., 1985).

Previous circular dichroism studies did not indicate differences in the secondary structure composition between the E\textsubscript{1} and E\textsubscript{2} states in sarcoplasmic reticulum (Nakamoto and Inesi, 1986; Csermely et al., 1987). It is difficult to list the specific reasons that may contribute to these differences between circular dichroism and FT-IR data. It is likely, however, that the greater information content, better signal-to-noise ratio, less interference by light scattering, and more sophisticated data analysis by Fourier transform infrared spectroscopy permitted the resolution of structural differences between the E\textsubscript{1} and E\textsubscript{2} states that remained undetected by circular dichroism, although the magnitude of the structural differences seen by FT-IR is difficult to quantitate.

Surprisingly, 20 mM Ca\textsuperscript{2+} produced similar changes in the vibrational modes assigned to protein secondary structure as vanadate, suggesting stabilization of the E\textsubscript{2} conformation at high Ca\textsuperscript{2+} concentration. Based on kinetic studies the Ca\textsuperscript{2+}-ATPase in the E\textsubscript{2} conformation has low affinity sites for Ca\textsuperscript{2+} (Inesi and de Meis, 1985). Therefore, it is plausible to assume that Ca\textsuperscript{2+} at concentrations sufficient to saturate low affinity binding sites (>20 mM) would shift the conformational equilibrium in favor of the E\textsubscript{1} form. Vanadate achieves the same effect by stabilizing an E\textsubscript{2} type intermediate at a different stage of the Ca\textsuperscript{2+} transport cycle. Such interpretation is supported by recent observations of Varga et al. (1986) that the irreversible denaturation of Ca\textsuperscript{2+}-ATPase at pressures of 1000–2000 atm can be prevented or slowed either by vanadate in a calcium-free medium or by high Ca\textsuperscript{2+} concentration; at similar pressures sarcoplasmic reticulum vesicles stripped from Ca\textsuperscript{2+} by EGTA completely and irreversibly lose their enzymatic activity within minutes. These observations also imply that the mixture of enzyme conformations that exist in a Ca\textsuperscript{2+}-free media, although usually considered to be predominantly E\textsubscript{2}, differs from the E\textsubscript{2} conformation stabilized in a Ca\textsuperscript{2+}-free system by vanadate.

There are significant differences between spectra obtained in the E\textsubscript{1} and E\textsubscript{2} state in the intensity ratio of the 1741 cm\textsuperscript{-1} and 1731 cm\textsuperscript{-1} bands that are assigned to the vibrational modes of the sn-1- and sn-2-ester carbonyl groups of phospholipids. This observation suggests a relationship between the conformation of the protein and that of the interfacial region of phospholipids. The methylene and methyl vibrations associated with the acyl chains of the phospholipids do not show such sensitivity.